PASSIVE IMMUNOTHERAPY AGAINST MALIGNANT MELANOMA

DESCRIPTION

BACKGROUND OF THE INVENTION

[0001] This invention relates to the use of antibodies for producing a vaccine for immunization against malignant melanoma, in particular for passive immunization against high molecular weight melanoma-associated antigen (HMW-MAA) – an important antigen – on melanomas, as well as the combination of antibodies and substances for producing a vaccine for treatment of malignant melanoma.

[0002] Antigens can be either exogenous (foreign) substances or endogenous substances (self-antigens). In the case of foreign antigens – usually proteins or polysaccharides – these are recognized by the immune system and, if they are classified as dangerous, eliminated by a corresponding immune response. The immune system, which has a non-specific and a specific line of action, acts by means of humoral and cellular factors. This specific cellular defense is mainly carried out through cytotoxic T cells, which either directly destroy a morbid agent or cells infected by pathogenic antigens, or the cells act through certain messengers which help other cells to eliminate the morbid substance or state.

[0003] The humoral factors of the specific immune system include antibodies which are produced by B cells or plasma cells. These antibodies are produced and secreted with the help of certain messengers, which in turn derive from T cells (T helper cells). The antibodies formed are specific to a certain region on the surface of an antigen, which is called the B cell epitope (there also being T cell epitopes, which are usually

linear and do not have the same localization as the B cell epitopes on the molecule's surface).

[0004] There are several possibilities for how an antibody can act.

[0005] An antibody fundamentally bonds with its specific binding sites, the idiotopes/paratopes, which are situated in the hypervariable region of the Fab part of the antibody, to specific regions of the antigen, which are called epitopes – this bond being effected according to the "lock and key principle".

[0006] This bond can neutralize an antigen and render it harmless; in the case of a proliferating cell, the antibody bond can stop (or also increase) the growth of the cell; due to the bound antibody the antigen can be absorbed more easily by cells (opsonization and phagocytosis by corresponding phagocytes, e.g. macrophages); or the bond of the antibody with its Fc region to effector cells (macrophages, NK cells) can activate these cells to be able to more easily attack and eliminate the target cells or target organisms.

[0007] Unlike foreign antigens, endogenous substances, cells, etc., are not seen to be foreign and dangerous. On the contrary, it is even desirable that these self antigens are tolerated by the immune system, since serious inflammations and tissue damage would otherwise constantly occur through the activation of the immune system (an example of misdirection of the immune system toward self antigen being autoimmune diseases).

[0008] When an overshooting growth of endogenous cells occurs – through a misdirection or failure of the regulatory system – as is the case with a tumor, the immune system does not react as a rule, since it has built up a tolerance toward endogenous proteins. Due to this fact, the tumor can spread in the body unhindered.

[0009] With the help of specific immunizations one can obtain a protective state against certain pathogens or morbid agents. In specific immunotherapy/immunization one distinguishes two types: active immunization – equivalent to a vaccination – by which substances (weakened pathogens, toxins, etc.) are supplied to the body to make the body build up protection itself. Attainment of the protective state takes several weeks, the protection can persist for several years, in some cases even for life.

[0010] In contrast, there is passive immunization (which is not a vaccination). Here, antibodies that are already formed are supplied to the body, the protection begins immediately upon application but does not persist very long, since the antibodies are broken down. By repeated administration of the antibodies the protective state can be maintained for a longer time.

[0011] In the case of tumor treatment, it is expedient to obtain a reduction of tumor growth with the help of passive immunization using antibodies aimed at a certain tumor antigen. This has been shown in the treatment of breast cancer with the application of the antibody trastuzumab in combination with a standard chemotherapeutic (McLaughlin, P. et al., J. Clin. Oncol. 16, 2825-2833 (1998)), or rituximab for treating relapses of non-Hodgkin's lymphoma (Baselga, J., Norton, L. et al. Cancer Research 58, 2825-2831 (1998)). The antibodies are humanized mouse antibodies (i.e. mice are immunized with human tumor cells and form specific antibodies against these foreign antigens, replacing the murine Fc region with a human one using molecular biological methods makes the humanized antibody better tolerated by humans).

[0012] Melanoma belongs to a group of very malignant, often therapy-resistant tumors, which can appear in 90% of cases without a genetic predisposition, and in 10%

with a family history. The incidence of cases of malignant melanoma has increased enormously within the past few years.

[0013] Only a stage I/II primary tumor, which has not passed through the basal membrane, can be cured by surgical removal of 85% of cases. In cases where tumor growth is > 1.5 mm or lymph nodes are already affected (stages III/IV), however, the prognosis is very bad, one reason being that in these stages there is an effective therapy in only an unsatisfactory low percentage (approx. 20%).

[0014] It has been shown that the aggressive growth of this tumor is associated with an abnormal protein expression. One of the most important of these melanoma antigens is HMW-MAA (high molecular weight melanoma-associated antigen), which is found on 90% of all primary and metastatic tumors. HMW-MAA is particularly responsible for the fast tumor growth and the tumor invasiveness.

[0015] Biochemical analyses have shown that HMW-MAA is a glycoprotein-proteoglycan complex, with a molecular size of > 450 kDa. This antigen shows strong immunogenicity, since it contains numerous epitopes against which different monoclonal mouse antibodies were produced.

[0016] One of these monoclonal antibodies is the 225.28S antibody, which was produced by immunization of BALB/c mice with the human melanoma cell line M21. The epitope which is recognized by the 225.28S antibody differs clearly in localization from those recognized by other monoclonal antibodies. The bound antibody is not endocytized and adheres to the membrane of the tumor cells.

[0017] A $F(ab)_2$ fragment of 225.28S which was conjugated to ^{99m}Tc (technetium) was used as an immunoconjugate (Technemab-K1; Sorin Biomedica, Italy) for

diagnosing melanoma. The article "Effects of Diagnostic Application of Monoclonal Antibody on Survival in Melanoma Patients" by H. Bender et al., pages 65-68, Hybridoma, Vol. 16, No. 1, 1997, Mary Ann Liebert, Inc., describes the coupling of the $F(ab)_2$ fragment of an anti-melanoma antibody to a radioactive substance (technetium), this radioactively labeled antibody fragment being used for diagnosing melanomas, or for diagnosing the tumor development. It turned out that a longer survival time was observed in persons in whom the labeled antibody fragment was used. It was furthermore shown that the tumor-reducing effect is usually to be ascribed to the radioconjugate used.

[0018] However, there is a great desire to do without radioconjugates in the treatment of patients. It is therefore of great interest to obtain a passive immunization without the use of conjugates if possible.

[0019] The problem of the present invention is therefore to find a way of treatment which is suitable for reducing or stopping melanoma growth and metastasis formation while largely doing without radioconjugates.

[0020] The invention is based on the finding of using antibodies suitable for passive immunization against malignant melanoma.

[0021] The invention is therefore to use antibodies suitable for producing a vaccine for passive immunization against high molecular weight melanoma-associated antigen on malignant melanomas.

[0022] High molecular weight melanoma-associated antigen (HMW-MAA) is found on 90% of all primary and metastatic tumors and is one of the most important melanoma antigens, since this antigen is associated with tumor spread and invasiveness.

The use of antibodies aimed at HMW-MAA is a particularly effective immunization, since this antigen is found in high number on malignant melanomas. It is particularly preferred that the antibodies used for producing a vaccine for passive immunization against HMW-MAA on malignant melanomas are monoclonal antibodies.

[0023] Furthermore, it is preferred that the antibody is the monoclonal antibody 225.28S. This monoclonal antibody is generated by immunizing (BALB/c) mice with human melanoma M21 cells. The monoclonal antibody 225.28S reacts strongly with melanoma cells which express HMW-MAA. For this reason the monoclonal antibody 225.28S is particularly well-suited for passive immunization against HMW-MAA on malignant melanomas. It has been shown by tests that the monoclonal antibody 225.28S strongly reduces the tumor growth of melanomas in vivo. It can thus be recognized from Figure 2 that the growth of human tumor cells is considerably lower in SCID mice receiving this antibody passively applied compared to tumor cells in mice treated with control antibody or saline solution.

[0024] It is thus described for the first time in this invention that intravenous applications of these antibodies lead to massive tumor reduction. Furthermore, it should be noted that this tumor reduction is obtained without additional administration of a chemotherapeutic (as has been described for other monoclonal antibodies against e.g. breast cancer).

[0025] Furthermore, it is preferred that the antibody is marker-free. Antibodies are used primarily for conjugating to markers and then using these conjugates for diagnostic purposes in tumors. Fluorescent and/or radioactive markers are preferably used here. The antibody in such a conjugate serves only to bring the active substance to

the site of action and bind it. In the present invention, however, it is preferred that the antibody serves as an active agent itself and thus acts directly on HMW-MAA.

[0026] Furthermore, it is preferred that the antibody is conjugate-free, i.e. that the antibody is not connected to a further substance. In the present invention, "connected" is understood to involve covalent or ionic bonds or other interactions, such as hydrogen bridge bonds or Van der Waals forces. It is in particular preferred that the monoclonal antibody 225.28S is nonconjugated.

[0027] Furthermore, it is preferred that the whole antibody is used for producing a vaccine for passive immunization against HMW-MAA on malignant melanomas. In particular, it is preferred that the whole antibody is the monoclonal antibody 225.28S.

[0028] Furthermore, it is also conceivable that fragments of antibodies can be used. In particular, it is preferred that $F(ab)_2$ fragments of antibodies are used for producing a vaccine for passive immunization against HMV-MAA on malignant melanomas. The F(ab) fragments are the parts of the antibody molecule that bond to the antigen (Fab = fragment antigen binding). The prototype of the antibody is built up symmetrically, consisting of four protein chains which are held together by noncovalent bonds and disulfide bridges. After breakage of these bonds there are two pairs of chains, which are referred to as heavy (H) and light (L) chains due to their different molecular weights. If the antibody is subjected to protolytic cleavage, however, this results in three fragments, two of which each consist of the L chain and the terminal ends of the H chain. These fragments are the above-mentioned F(ab') fragments.

[0029] It is in particular preferred that $F(ab)_2$ fragments of the monoclonal antibody 225.28S are used.

[0030] Furthermore, it is preferred that the antibody, in particular a monoclonal antibody and more particularly the monoclonal antibody 225.28S, is used with other active substances acting on HMW-MAA for producing a vaccine for passive immunization against HMW-MAA on malignant melanomas.

[0031] The active substances are preferably chemotherapeutics. It is particularly preferred to use chemotherapy cytostatics, most preferred are decarbazines (DTIC), temozolamides, cisplatin, fotemustines (Muphoran) and vincristines-venblastines.

[0032] In particular, it is preferred that the active substances are monoclonal antibodies, preferably antibodies that bind HMW-MAA.

[0033] Furthermore, it is preferred that these further monoclonal antibodies bind to different epitopes of high molecular weight melanoma-associated antigen compared to the monoclonal antibody 225.28S.

[0034] The invention will hereinafter be explained more closely by examples.

EXAMPLES

Animals

[0035] Six week-old pathogen-free CB-17 scid/scid mice, obtained from Harlan Winckelmann, Germany, and kept in microisolator cages, were given autoclaved food and water ad libitum during the experimental phase. All experiments were approved by the Ethical Commission on Animal Experimentation of the University of Vienna and the Ministry of Development, Research and Culture.

Cell line and antibodies

[0036] The human melanoma cell line 518A2 (from Dr. Peter Schrier, Leiden, Netherlands) was kept in a DMEM medium (Life Techonolgies, Carlsbad, CA), provided with 10% fetal calf serum and 1% antibodies in a 5% CO_2 and 95% humidified air atmosphere at 37°C. The cell culture was free from mycoplasma and pathogenic virus. The monoclonal mouse antibody 225.28S was provided by Soldano Ferrone. The monoclonal mouse IgG antibody clone LC1, which serves as a control antibody, was purchased from NeoMarkers, Fremont, CA.

Human melanoma SCID xenotransplantation model

Experimental procedure (Fig. 1)

[0037] 1×10^7 of the human melanoma cell line 518A2 resuspended in 200 µl of sterile PBS was injected subcutaneously into the left flank of the mouse. Approx. 14 days later, after the tumors had reached an average diameter of 5 mm, the vaccine was administered intravenously.

[0038] The mice were divided up into three groups of five mice each. One group was given monoclonal antibody 225.28S, the second group was given a control antibody (mouse IgG), and the third group had only saline solution (untreated) applied. 100 µg of the particular antibody (i.e. 5 mg/kg body weight) was given intravenously in a volume of 250 µl four times at 3-day intervals. Tumor size was determined by calibration measurement two times a week. Tumor volume was calculated according to the following formula: volume = (longest tumor diameter x shortest tumor diameter^2)/2. Five

days after the last injection, the mice were killed. The tumors were exposed and their weight measured. The experiments were repeated three times under the same conditions.

Results

Reduction of tumor growth by administration of monoclonal antibody 225.28S in vivo

[0039] To study the biological activity of monoclonal antibodies 225.28S in vivo, the monoclonal antibody was administered four times at an interval of three days in SCID mice (immune-incompetent mice lacking functional T and B lymphocytes) with an established human melanoma. After three intravenous applications of monoclonal antibodies 225.28S a significant reduction of tumor volume could already be ascertained in the mice treated with monoclonal antibodies in comparison with the untreated mice (placebo-treated with sodium chloride solution) (Fig. 2). After the third antibody treatment, a smaller tumor volume was likewise ascertained compared to the mice treated with a non-specific control antibody. Five days after the last antibody treatment, the tumor volume of the mice treated with monoclonal antibody was 50% smaller in comparison with the mice treated with control antibody as well as the untreated control mice (treated with sodium chloride).

[0040] At this time the tumor weight was also determined, which was likewise 50% lower than in the control mice (Fig. 3). The experiments were carried out three times under the same conditions, whereby the shown data render the average of the three series of experiments.

Statistics

[0041] The date of all three independent experiments were evaluated statistically using variance analysis and contrast analysis (* = $P < 0.05$; ** = $P < 0.01$). Five mice/group were used per experiment.

Experimental procedure (cf. Fig. 1)

[0042] The experiment was started by subcutaneous injection of 1×10^7 melanoma cells (518A2) into the flanks of the mice (d0). Fourteen days later, after the diameter of the tumors had reached an average of 5 mm, the first intravenous injection of monoclonal antibody 225.28S (100 µg / 250 µl) was injected, or control antibody (100 µg / 250 µl) or sodium chloride solution (sodium chloride, 250 µl) administered. Treatment was carried out four times at a time interval of three days (d14, 17, 20, 23) and then, after each administration of antibody, the tumor size measured. Five days after the last administration of antibody the mice were killed (d28) and the tumors exposed and weighed.

Tumor Volume (cf. Fig. 2)

[0043] Fourteen days after the inoculation of the tumor cells (d10), after the average diameter of the tumors had reached 5 mm, the first intravenous injection of monoclonal antibody 225.28S (100 µg / 250 µl) or the injection of control antibody (100 µg / 250 µl) or the injection of sodium chloride (sodium chloride, 250 µ) was carried out. Treatment was carried out after 14, 17, 20 and 23 days. Five days later (d28) the mice were killed. After each antibody application and after the end of the experiment the tumor volume was evaluated. After the third application of 50% reduction of tumor

volume could already be ascertained compared to the control antibody treated or sodium chloride treated mice (* = $P < 0.5$; ** = $P < 0.01$).

Tumor weight (cf. Fig. 3)

[0044] After 28 days the animals were killed and the tumor exposed and weighed. In accordance with the reduced tumor volume, tumor weight was likewise 50% reduced after treatment with the monoclonal antibodies 225.28S over that of the control animals.